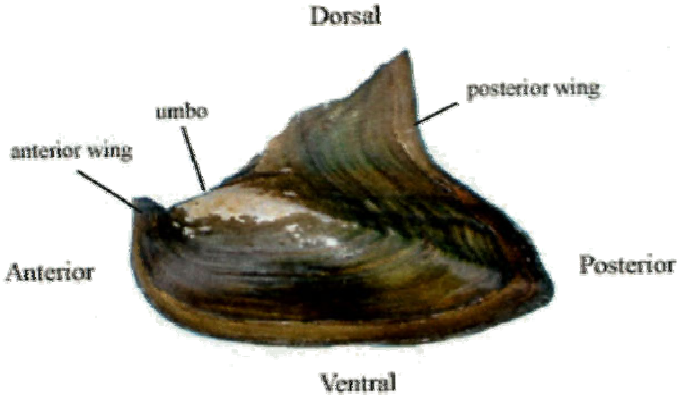
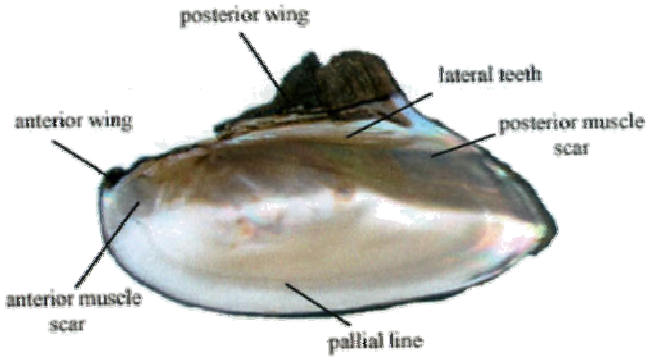
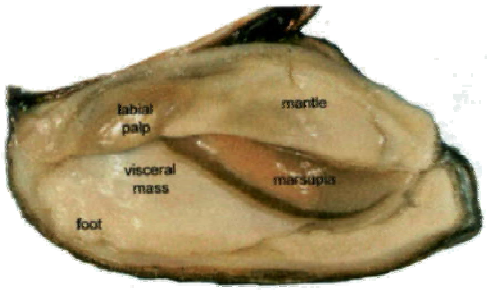
APPENDIX



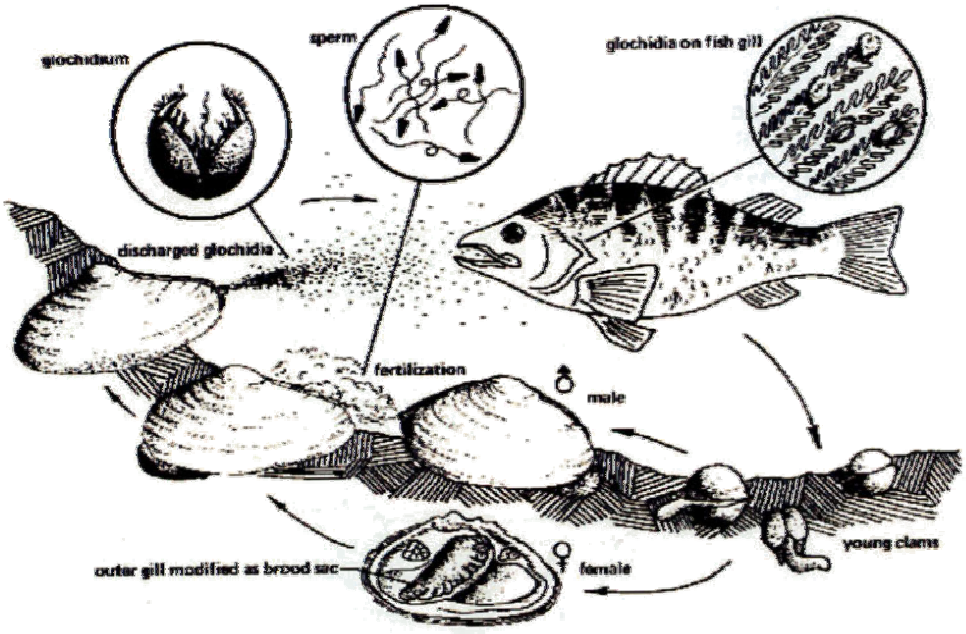
Appendix Figure 1 Outer shell appearance of *Hyriopsis (Hyriopsis) bialatus*



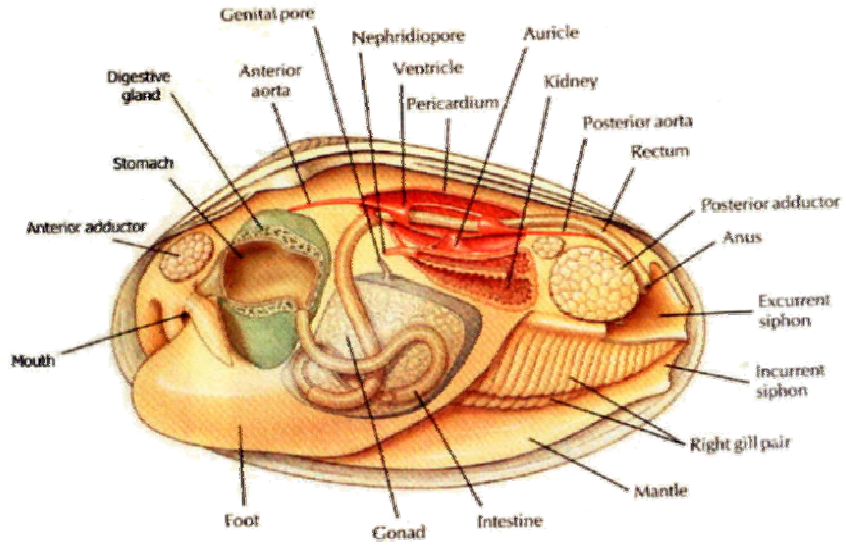
Appendix Figure 2 Inner shell appearance of *Hyriopsis (Hyriopsis)bialatus*



Appendix Figure 3 Anatomy of *Hyriopsis (Hyriopsis)bialatus*



Appendix Figure 4 The life cycle of Freshwater pearl mussels
Source: Wisconsin, 2005



Appendix Figure 5 The diagram illustrating an anatomic structure of mussels.
Source: Hickman and Roberts (1995)

Appendix Table 1. Comparison of glochidial dimensions of several unionids.

Species	Size (μm)			References
	Length	Height	Width	
Anodonta arcaeformis	470	400	-	Jeong *et al*. (1992)
Hyridella depressa	243	249	-	Jupiter and Byrne (1997)
Hyriopsis myersiana	200	150	75	Kovitvadhi *et al*. (2001)
H. (H.) bialatus	190±0.02	230±0.02	61±0.86	This study
Margaritifera auricularia	127-144	120-142	54-71	Arajoa and Ramos (1998)
M. laevis	70-90	-	-	Awakura (1968)
M. falcata	50-73	75-80	-	Murphy (1942), Karna and Millemann (1978)
M. margaritifera	50-70	70-80	-	Harms (1909), Bykhovskaya-Pavlovskaya *et al*. (1964), Smith (1976), Young and Williams (1984), Nezlin *et al*. (1994), Bauer (1994), and Pekkarinen and Valovirta (1996)
Cumberlandia monodonta	55	-	-	Howard (1915)

Source: Modified from Arajo and Ramos (1998)

Preparation of glochidia medium

1. Fish plasma preparation

Eight to ten mL of blood was extract with a syringes with an 18-gauge (1.2 mm in diameter, 40 mm in length) from the caudal vein of each common carp fish (*Cyprinus carprio*). The syringes were coated with sodium heparin at 1000 units per mL concentration. The blood was centrifuged at 1000 and 3000 g for 10 min each. The supernatant was collected and placed into a new test tube and centrifuged again at 3000 g for 10 min. The plasma portion (clear yellow solution) was separated and filtered through 0.45 μm and 0.25 μm filter paper, respectively. The plasma was stored at -20 ^{o}C for further media preparation.

2. M 199 preparation

A packet of M 199 powder (Life Technologies, No. 71N0262) was dissolved in distilled water to make the total volume of 1 l, 2.0 g of $NaHCO_3$ was add, and the pH was adjusted to 7.3-7.4 by titration with 1 M NaOH.

3. Culture medium preparation

M 199 solution was mixed with fish plasma and antibiotics (carbenicillin, gentamycin and rifampin) plus antimycitic (amphotericin) at the ratio of 2:1:0.5 by volume(Appendix Tables 2 and 3) and filtered using 0.45 and 0.22 μm filter paper and kept at -80 ^{o}C for further use.

Appendix Table 2. Composition of artificial medium for *in vitro* culture of glochidia *Hyriopsis bialatus*.

Composition of media	Concentration of artificial medium (mL)
M199	2.0
Fish (*Cyprinus carpio*) plasma	1.0
Antibiotics and antimycotic*	0.5

* Details in Appendix Table 3.

Source: Isom and Hudson (1982, 1984a, and 1984b), Keller and Zam (1990) and modified by Kovitvadhi *et al.* (2001).

Appendix Table 3. Antibiotics and antimycotic chemicals for culture *in vitro* culture of glochidia *Hyriopsis bialatus*.

Compound	Concentration (μg/L)
Antibiotics	
Carbenicillin	100
Gentamycin sulfate	100
Rifampin	100
Antimycotic	
Amphotericin B	5

Source: Isom and Hudson (1982, 1984a, and 1984b), Keller and Zam (1990)

Histological procedure

1. Tissue processing

1.1 ***Fixation*** Fixation was done with 10% neutral buffer formalin for 2 h and preserved in 5% neutral buffer formalin.

1.2 ***Dehydration*** The dehydration process continues by rinsing a series of ethanol at 10%, 30%, 50% and 70% for 10 min each, in 95% ethanol for 10 min twice, in absolute ethanol for 15 min twice.

1.3 ***Clearing*** Clearing was done with xylene for 5 min twice. Xylene is use as the clearing reagent. As the dehydration is removed, the tissues are cleared, becoming translucent signifying. This process is completely.

1.4 ***Infiltration*** Infiltration was done twice in melted paraplast for 15 min at 60 ^{o}C in the hot air oven.

1.5 ***Embedding*** The samples were oriented in the block with melted paraplast. Vortex the block to distribute the samples randomly and thoroughly, then floating the tissue block on cold water immediately. This process is required for fixing the irregular plane and position of numerous minute samples in each tissue block.

1.6 ***Sectioning*** The various steps in sectioning tissue. Set the rotary microtome to the appropriate level (5-6 µm). Cutting block with disposable microtome blade. The ribbon of sections placed in shallow box. Immediate floating the section on the warm water bath with gelatin adhesive. Gelatin adhesive provide a filmer attach of the section. Picking up the section on a microscopic slide. Drying sections on a hot plate at 37 ^{o}C overnight or at least 3 h.

2. Staining procedure

2.1 Harris's hematoxylin and eosin stain

1. Deparffinization section in xylene, two changes of 6 minutes each.
2. Dip in absolute alcohol two changes of 2 min each.
3. Place in 95% ethyl alcohol and then in 70% for 2 min each.
4. Hydrate section in distilled water for 5 min.
5. Staining in Harris's hematoxylin for 6 minute. Rinse in tap water for 5 min.
6. Differentiate in acid alcohol, 3 quick dips. Nuclei should be distinct and the background very light or colorless.
7. Wash in tap water for 5 min.
8. Dip in ammonia water until sections are bright blue, (3 to 5 dips).
9. Wash in running tap water for 1-2 min.
10. Stain with eosin for 30 sec.
11. Dehydrate in 95% alcohol two changes of 2 min each.
12. Absolute alcohol, two changes of 2 min each.
13. Clearing in xylene, two changes of 5 min each.
14. Mount with Permount.

Result:	Nuclei	-	blue
	Cytoplasm	-	Various shade of pink
	Connective tissue	-	pink-red

2.2 Masson's trichrome stain

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Bouin's solution for 1 h at 56 ^{o}C, or overnight at room temperature, if formalin fixed.
3. Cool and wash in running water until yellow color disappears.
4. Rinse in distilled water.
5. Weigert's iron hematoxylin solution for 10 min. Wash in running water 10 min.
6. Rinse in distilled water.
7. Biebrich scarlet-acid fuschin solution for 2 min. Save solution.
8. Rinse in distilled water.
9. Phosphomolybdic-phosphotungstic acid solution for 10 to 15 min before aniline blue solution. (Aqueous phosphotungstic acid 5% for 15 min before light green counterstain). Discard solution.
10. Aniline blue solution for 5 min or light green solution for 1 min. (for central nervous system 15 to 20 min) Save solution.
11. Rinse in distilled water.
12. Glacial acetic acid solution for 3 to 5 min. Discard solution.
13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
14. Mount with Permount.

Result:

Nuclei	-	black
Cytoplasm, keratin, muscle fibers and intercellular fibers	-	red
Collagen, mucopolysaccharide, elastic fibers	-	blue

2.3 Perriodic Acid Schiff reaction (PAS) stain

1. Hydrate slides
2. Immerse in periodic acid for 5 min. Discard solution.
3. Rinse in several changes of distilled water.
4. Immerse in Schiff reagent for 15 min. Filter back.
5. Rinse slide in sulfurous rinse, 3 changes of 2 min each. Discard each change.
6. Wash in running tap water for 10 min.
7. Counterstain in Harris's hematoxylin for 1 min. Filter back.
8. Wash in running tap water for 10 min.
9. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
10. Mount with Permount.

Result:	Nuclei	-	blue
	Neutral mucopolysaccharide	-	magenta

Preparation of histological reagent

1. Fixatives

1.1 Neutral Buffer Formalin (NBF)

37- 40% formalin	100.0	ml
Distilled water	900.0	ml
Sodium phosphate monobasic	4.0	g
Sodium phosphate dibasic	6.5	g

1.2 Bouin's fluid

Saturated aqueous picric acid solution	75	ml
40% formaldehyde	25	ml
Glacial acetic acid	5	ml

2. Staining reagents

2.1 Harris's hematoxylin and eosin stain

2.1.1 Harris's hematoxylin

Hematoxylin crystals	5.0	g
Absolute alcohol	50.0	ml
Aluminum ammonium sulfate	100.0	g
Distilled water	1000.0	ml
Mercuric oxide	2.5	g

Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat. Removal from heat and mix the two solutions. Bring to a boil

as rapidly as possible. (less than 1 min) Remove from heat and add the mercuric oxide slowly. Reheat to a simmer until it becomes dark purple, remove from heat immediately and plunge the vessel into a basin of cold water until cool. The stain is ready for use as soon as it cools. Addition of 2-4 ml of glacial acetic acid per 100 ml of solution increases the nuclear stain. Filter before use.

2.1.2 Eosin-Phloxine solution

Stock Eosin:

Eosin Y, water soluble	1.0	g
Distilled water	100.0	ml

Stock Phloxine

Phloxine B	1.0	g
Distilled water	100.0	ml

Working solution

Stock Eosin	100.0	ml
Stock Phloxine	10.0	ml
Alcohol, 95%	780.0	ml
Glacial acetic acid	4.0	ml

Make up working solution as needed. Working solution should be changed at least once a week.

2.1.3 1% acid alcohol

70% Ethyl alcohol	1000.0	ml
HCL, concentrated	10.0	ml

2.1.4 Ammonium water

Ammonium	150.0	g
Distilled water	1000.0	ml

2.2 Masson's trichrome stain

2.2.1 Weigert's iron hematoxylin solution

Solution A:

Hematoxylin crystals	1.0	g
Alcohol, 95%	100.0	ml

Solution B:

Ferric Chloride, 29% aqueous	4.0	ml
Distilled water	95.0	ml
Hydrochloric acid, concentrated	1.0	ml

Working solution:

Equal parts of Solution A and Solution B.

2.2.2 Biebrich scarlet-acid fuchsin solution

Biebich scarlet, aqueous, 1%	90.0	ml
Acid fuchsin, aqueous, 1%	10.0	ml
Glacial acetic acid	1.0	ml

2.2.3 Phosphomolybdic-phosphotungstic acid solution

Phosphomolybdic acid	5.0	g
Phosphotungstic acid	5.0	g
Distilled water	200.0	ml

2.2.4 Aniline blue solution

Aniline blue	2.5	g
Glacial acetic acid	2.0	ml
Distilled water	200.0	ml

2.2.5 1% Glacial acetic acid solution

Glacial acetic acid	1.0	ml
Distilled water	100.0	ml

2.3 Periodic Acid Schiff (PAS) reaction stain

2.3.1 1% Periodic acid solution

Periodic acid	1.0	g
Distilled water	100.0	ml

2.3.2 Schiff reagent solution

Basic fuchsin	1.0	g
Potassium metabisulfite	2.0	g
Normal hydrochloric acid	10.0	ml
Activated carbon	0.5	g
Distilled water	200.0	ml

Dissolve 1.0 g basic fuchsin in 200.0 ml hot distilled water. Bring to boiling point. Cool and add 2.0 g potassium metabisulfite, 10.0 ml normal hydrochloric acid. Let bleach for 24 h, and then add 0.5 g activated carbon. Shake for 1 min and filter through coarse filter paper. Repeat filtration until solution is colorless. *Store in refrigerator*.